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Short Communication

Identification of novel *brown midrib* genes in maize by tests of allelism

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With 2 tables

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Abstract

Brown midrib (*bm*) mutations are known to affect cell-wall digestibility by altering the quantity and composition of lignins in cell walls, resulting in higher ethanol yield and increased cell-wall digestibility. So far, four *bm* genes (*bm*1, *bm*2, *bm*3 and *bm*4) were identified and mapped in maize, the last one (*bm*4) in 1947. In this study, 13 spontaneous mutations (*bm**A–M) resulting in the appearance of brown midribs were crossed with *bm*1–4 for tests of allelism. From these tests, we report two new *bm* mutants *bm*5 (*bm**F) and *bm*6 (*bm**J) while other *bm** lines were either found allelic to *bm*1–4 or to one of the *bm** lines.

Key words: bm gene—allele test—maize—ethanol—lignocelluloses—biomass

Maize (*Zea mays*) is one of the most important cereal and fodder crops world-wide. Maize fulfils 15–20% of the daily calorie needs of people in more than 20 countries in the world (Dowswell et al. 1996). Due to high biomass accumulation, maize stover is also a good source for forage and bio-fuel production.

Globally, energy security is one of the major concerns besides food security. Due to the non-renewable nature of traditional sources of energy (oil, coal, natural gas), efforts are being made to find other avenues for alternative sources of energy, which are both clean and low cost. Ethanol has emerged to be one of the most important alternate energy resources, especially in USA and Brazil, which are two of the major ethanol producing countries. Global ethanol production in 2009 is expected to be 17.5 billion gallons (World's Ethanol Production Forecast 2008–2012) which is exclusively based on grain, while efforts are being made to utilize lignocellulosic materials for production of ethanol that will not only be more cost efficient, but in addition will not inflate prices of food commodities.

One of the major constraints in ethanol production is the conversion efficiency of biomass into ethanol. The goal is to increase the total amount of available digestible sugars, which is limited by cell-wall lignification. *Brown midrib* (*bm*) mutants have been shown to increase glucose yield, while reducing the lignin content of cell walls, yielding more ethanol per unit of biomass (Dien et al. 2009). In addition, *bm* mutants are known to increase the cell-wall digestibility by altering lignin composition and reducing the lignin content of cell walls, thus improving the nutritional value of silage in maize and Sudangrass, when used as a fodder crop (Cherney et al. 1991, Campbell and Sederof 1996, Casler et al. 2003).

The first *bm* mutant was observed in dent corn at Saint Paul, Minnesota in 1924 (Lauer and Coors 1997). So far, four *bm* mutations have been characterized: *bm*1 (Jorgenson 1931), *bm*2 (Burnham and Brink 1932), *bm*3 (Emerson et al. 1935) and *bm*4 (Burnham 1947). These mutants show reddish brown pigmentation in leaf midribs. Besides leaves this reddish brown pigmentation is also visible in stalk pith after the plant has attained five expanded leaves (Lauer and Coors 1997, Barriere et al. 2004). Colouring eventually disappears on leaves, but remains in the stalk (Lauer and Coors 1997). Biochemical comparison of these mutants with respective wild types showed not only reduced lignin content in *bm* mutants, but also changes in composition of lignin and cell-wall digestibility (Barriere and Argillier 1993). Out of the four *bm* mutants (*bm*1–4), *bm*3 strongly affects the phenotypic appearance and is reported to improve cell-wall digestibility by 16% in comparison to isogenic lines (Barriere and Argillier 1993). The *bm*2 mutation affects tissue specific patterns of lignification (Vermerris and Boon 2001) and has a similar phenotype as that of *bm*4 (Marita et al. 2003). The *bm*1 mutation results from differential expression of the cinnamyl alcohol dehydrogenase (CAD) gene (Halpin et al. 1998) while the *bm*3 mutation results from structural changes in the caffeic acid *O*-methyltransferase (COMT) gene (Vignols et al. 1995). *bm* mutations are also known in sorghum, Sudangrass and pearl millet (Lauer and Coors 1997).

Beside the four known *bm* mutations (i.e., *bm*1, *bm*2, *bm*3, and *bm*4), additional *bm* mutations have been identified, which were not further characterized and named *bm** mutants. Currently there are thirteen such mutant lines (Maize Genetic Stock Center Designations 5803A–M; abbreviated here *bm**A–M) available in the Maize Stock Centre (Maize Genetic Coop Centre, http://maizecoop.cropsci.uiuc.edu/). These spontaneous mutations were not previously tested against the already known *bm*1–4 mutations by tests of allelism to examine, whether these mutations are allelic to the already identified *bm*1–4 mutations, or these are mutations in different genes. The main aim of the current study was to test these *bm** (*bm**A–M) mutations by tests of allelism against the known *bm* mutants (i.e. *bm*1–4) to identify possible new *bm* genes.

The procedure used for the test of allelism was the same used by Koncz et al. (1990). Midrib colour in maize leaf is a qualitative trait where green/white midrib (wild type) is dominant over brown midribs of *bm* mutants. Thus, a cross of a homozygous *bm** line with any homozygous line carrying *bm*1, 2, 3 or 4 results in either 100% wild type (green midrib) plants, or 100% brown midrib plants indicating that a particular *bm** mutation is allelic to either *bm*1, 2, 3 or 4. For most of the mutations, results of the tests of allelism were clear-cut, but for mutations with weaker phenotypes (*bm**E and *bm**H), tests were replicated various times for validation. Seed received for *bm**M was segregating for the brown midrib phenotype. Therefore, only plants showing *bm* phenotype were used for the test of allelism. After testing all *bm** mutations against *bm*1–4, tests of allelism were performed among *bm** mutations, which differed from *bm*1–4.

A total of 13 novel *bm** mutant lines were provided by the Maize Genetic Stock Centre. Seed from these mutant lines was sown in two rows in the field. Crosses were made between *bm** and *bm*1–4 lines for tests of allelism. Due to difference in timing to maturity, crosses were made in both directions using both *bm** lines and *bm*1–4 as male and female plants to carry out all desired crosses at the Agronomy farm of Iowa State University, USA. Those *bm** lines which were identified as non-allelic to *bm*1–4 were crossed amongst themselves to test, whether these mutants are different from one another or allelic. For each cross, three pollinations were made and seed was harvested separately. After drying, seed for each cross was kept in separate bags. A total of 20 kernels (seven kernels from each bag per cross) from all crosses were sown in pots in the greenhouse. A minimum of 15 plants per cross were used for scoring. Plants were allowed to grow until appearance of the brown midrib phenotype or maturity, whichever came earlier. The data for Table 1 were collected over a period of 2 years in the field and greenhouse and tests were replicated in independent trials. Some of the tests of allelism were performed independently by collaborating scientists, Sarah Hake (USDA, personal communication) and Paul Scott (earlier results obtained prior to this study), and the results were generally in agreement with our findings. In case of discrepancies, additional tests of allelism were performed. Further characterization of the novel brown midrib mutants as isogenic line as well as genetic mapping is ongoing.

*bm**A and *bm**B have radically different phenotypes than all other *bm* mutations (Table 1). They were, therefore, excluded from further analyses. *bm**C, *bm**D and *bm**L were allelic to *bm*1. All F1 plants of these mutants with *bm*1 resulted in appearance of brown midribs (Table 1). *bm**K was allelic to *bm*3, and *bm**M to *bm*2. *bm**E, *bm**F, *bm**G, *bm**H and *bm**J were non-allelic to *bm*1–4, and were, therefore, crossed among each other for additional tests of allelism to determine the number of independent and novel *bm* mutations (Table 2).

Table 1: Results of tests of allelism between *bm** (*bm**A–M) mutations and *bm*1–4

	*bm*1	*bm*2	*bm*3	*bm*4	Remarks	Stock ID
*bm**A					Shootless, not studied	5803A
*bm**B					Bladeless, not studied	5803B
*bm**C	+	−	−	−	*bm*1	5803C
*bm**D	+	−	−	−	*bm*1	5803D
*bm**E	−	−	−	−	Not *bm*1–4	5803E
*bm**F	−	−	−	−	Not *bm*1–4	5803F
*bm**G	−	−	−	−	Not *bm*1–4	5803G
*bm**H	−	−	−	−	Not *bm*1–4	5803H
*bm**J	−	−	−	−	Not *bm*1–4	5803J
*bm**K	−	−	+	−	*bm*3	5803K
*bm**L	+	−	−	−	*bm*1	5803L
*bm**M	−	+	−	−	*bm*2	5803M

+Allelic; −non-allelic.

Table 2: Tests of allelism among *bm** mutants different from *bm*1–4

	*bm**E	*bm**F	*bm**G	*bm**H	*bm**J	Summary
*bm**E						*bm*5
*bm**F	+					*bm*5
*bm**G	+	+				*bm*5
*bm**H	+	+	+			*bm*5
*bm**J	−	−	−	−		*bm*6

+Allelic; −non-allelic.

The candidate mutations (i.e. *bm**E, *bm**F, *bm**G, *bm**H and *bm**J) for novel *bm* genes were crossed pair wise, to ascertain whether these are allelic or distinct *bm* mutants (Table 2). These results identified two novel *bm* genes. We designate *bm**F and *bm**J, as *bm*5 and *bm*6, while *bm**E, *bm**G, and *bm**H were all found allelic to *bm**F.

Four *bm* mutations (i.e. *bm*1–4) have been known for a long time. These mutant lines were introgressed into the same background to evaluate their effect on different agronomic parameters. These mutants have shown a drastic effect on cell-wall digestibility by altering lignin content and composition resulting in higher yield of total fermentable sugars (Lechtenberg et al. 1972, Barriere and Argillier 1993, Vermerris and Boon 2001). According to US Department of Energy, a 1% increase in total available fermentable sugar will result in 2.21×10^9 more gallons of ethanol for the same amount of biomass (Department of Energy). Double mutants were previously used and have shown an additive effect on total available fermentable sugars. For the *bm*1*bm*3 double mutant, total glucose yield obtained after enzymatic saccharification was twice as high as that of the A619 isogenic parent (Vermerris et al. 2007). These two new mutants may prove to be a valuable resource for development of highly convertible maize stover. Further characterization of the novel brown midrib mutants as isogenic line as well as genetic mapping is ongoing.

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